Western Blot to Examine Yeast Strains for Protein Expression

Sample preparation for protein gel:
(modified from Kushnirov, Rapid and reliable protein extraction from Yeast. Yeast 2000; 16: 857±860)

1. Spin down yeast culture that has been grown overnight in YPD.
2. Resuspend cells in 100 ul of water and add 100 ul of 0.2M NaOH
3. Incubate for 5 minutes at room temperature and then pellet the cells by centrifugation
4. Remove supernatant and Resuspend cells in 50ul of SDS Sample Buffer (2x)
5. Boil for 3 minutes and then centrifuge to pellet the cells
6. Move the supernatant to a new tube and label with the sample information. Throw away the pellet.
7. Load 15-20ul of supernatant per lane on a protein gel
8. Load 10ul of prestained protein ladder
9. Run protein gel at 200V until dye front reaches the bottom of the gel

Note: Be sure to either run two identical gels or load your samples in duplicate on one gel. You will use one set of samples for the western blot and the other for coomassie staining to check for protein extraction

Gel Removal

1. After electrophoresis is complete, turn off the power supply and disconnect the electrodes.
2. Remove the tank lid and carefully lift out the inner chamber assembly. Pour off the running buffer into a large beaker.
3. Remove the gel from the plates by using a metal spatula to pry the two plates apart. Allow the gel to stick to one of the plates.
4. At this point do not touch the gel. It is very sticky and will stick to your gloves. Soak the gel in a container with ~200 ml ddH2O for 5 minutes.
5. Repeat with the second gel in a different container. (or if using one gel, rehydrate and then cut gel in half with a clean razor blade)
6. After the gels have rehydrated, one will be used for Coomassie staining and the other will be used for Western blotting. Cut the gel with a razor blade to trim it down to a smaller size. Be sure to remove the wells from the top and the bump at the bottom.

Coomassie Staining

1. Take one of your gels and repeat the water wash one more time to remove the SDS from the gel.
2. Remove all water from the staining container and add enough Bio-Safe Coomassie stain (Bio-Rad Laboratories) to just cover the gel.
3. Gently rock on a rotating platform overnight.
4. Gels will be rinsed for you the next day. We will wash three times in ddH2O for 30 minutes each. Stained gels will be stored in water. You can take a picture of your gel during the next lab period.

Western Blotting

1. Place the second gel in Western Transfer buffer.

2. Soak the filter paper and nitrocellulose membrane in transfer buffer before assembling the "sandwich" (see picture below).
3. After the apparatus is assembled, the proteins will be transferred from your gel to the nitrocellulose membrane for 20 minutes at 20 Volts.
4. Rinse the membrane to remove any excess gel pieces by incubating in PBS for 10 minutes.
5. Block the membrane by placing in fresh blocking buffer overnight at 4degC with shaking.
6. **Wash the membrane in PBS (5 minutes)**
7. **Add your primary antibody diluted in blocking buffer**. Incubate for 1 hour at room temperature**
8. **Wash 3 x 10 minutes in wash buffer (PBS-T)**
9. **Add secondary antibody (conjugated to HRP) diluted in blocking buffer##. Incubate for 1 hour at room temperature**
10. **Wash 3 x 10 minutes in wash buffer (PBS-T)**
11. **Add CN-DAB substrate to develop the western. Mix the two reagents during your last wash from step 10.**
12. **Be ready with a waste container to pour off the substrate (not down the DRAIN!) and water to stop the development reaction**
13. **Immediately take a picture of the membrane.**

Western Transfer Buffer

For 2 liters:
125mM Tris (6.06g)
192 mM glycine (28.8g)
20% methanol (400ml) **Note: If using 95% (Tech) methanol, add 421ml of methanol.**
To 2 liters final volume with DI water
Transfer mini gels for 15–30 minutes at 10–15 Volts.

SDS-PAGE Running Buffer

10X Running buffer (also called Laemmli buffer):
Tris base 30.3g
Glycine 144g
SDS 10g
make to 1L with dH2O

10X PBS	500mL
NaCl	40g
KCl	1g
Na_2PO_4	7.2g
KH_2PO_4	1.2g
pH to 7.5	

Blocking Buffer
5% milk in PBS (5g in 100 ml usually is enough).

Wash Buffer
1X PBS with 0.1% Tween-20

Note: For this experiment we will spin down different amounts of yeast cells that have been grown overnight in YPD. Be sure to note down how long the cells have been growing and how much of the culture is used in each sample.

**Primary Antibody – anti-GFP rabbit antibody, http://tools.lifetechnologies.com/content/sfs/manuals/MP06455_AntiGFPAntibodies_PI.pdf
**Recommended dilutin is 1:1000. So can typically use 10ul in 10ml of Blocking Buffer. However, this is something we might try and adjust over time to improve the western blot.

##Secondary Antibody - Goat anti-Rabbit, HRP conjugate
##Recommended dilution is 1:300. So we can use 3ul in 9ml of Blocking Buffer. This is also something we can try and alter in the protocol to improve the signal.